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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
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Yasuki Kato, et al.)
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Serial No. 09/869,049)
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Pharmaceutical preparations)

Group Art Unit: 1651

Examiner: Brett T. Ozga

DECLARATION

The Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Yasuki Kato of 2-8-7, Senpukugaoka, Susono-shi, Shizuoka-ken, Japan, do declare as follows:

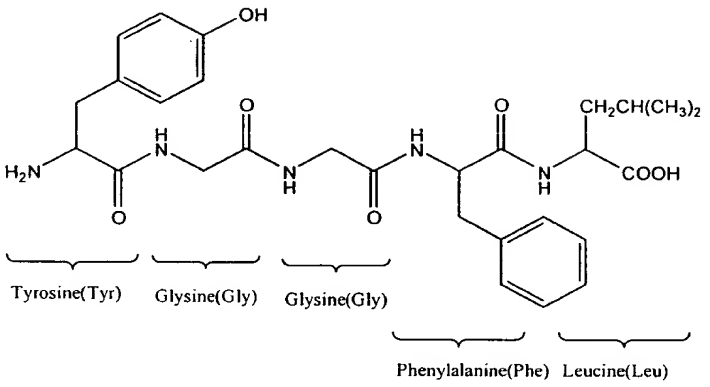
I finished my master course at Department of Applied Chemistry, Faculty of Technology, Gunma University in March, 1980, and I was given the degree of M.A. Since April, 1980, I have been employed by Kyowa Hakko Kogyo Co., Ltd., the assignee of the above-identified application. Since April, 1980, I have been engaged in the research of formulation and drug delivery systems at Pharmaceutical Research Institute of the company. I was given the degree of Ph.D. in 1993 from Faculty of Pharmaceutical Science, the University of Tokyo. From September 1993 to August 1994, I studied gene therapy using liposomes at Biopharmaceutical Sciences, School of Pharmacy, University of California in San Francisco.

The following experiment was conducted under my direction.

Experiment:

Example A:

The structure of Enkephalin (Leucine-Enkephalin, Peptide Institute, Inc.) is shown below.



Leucine-enkephalin

Enkephalin (0.53 mg) was dissolved in 0.53 mL of distilled water (the enkephalin solution). β-lactose (Nacalai Tesque, Inc.) was dissolved in distilled water to make a 200 mg/mL solution (the lactose solution). The enkephalin solution (0.06 mL), the lactose solution (0.3 mL), 0.15 mL of a 200 mmol/L phosphate-citric acid buffer (pH 8.0), and 0.09 mL of distilled water were mixed in a test tube to give a mixture having a pH of about 7.9. The test tube was put into a constant temperature water bath at 40°C to cause a reaction of enkephalin with lactose. The reaction mixture was sampled after 0, 5 and 24 hours, and analyzed for reaction products by high performance liquid chromatography (HPLC) under the following conditions.

Column: CAPCELL PAK C18 UG120, 4.6 x 250 mm (Shiseido Co., Ltd.)
Mobile phase:
a 50 mmol/L phosphate buffer (pH 7.3) : acetonitrile = 83 parts by volume : 17 parts by volume
Flow rate: 1.8 mL/minute
Detection wavelength: 210 nm

As a result, it was found that one reaction product was formed by the reaction of enkephalin with lactose. The reaction product was designated as Product-A. The retention time of Product-A was shorter than that of enkephalin. The results are shown in Table A.

Table A
Addition of Lactose to Enkephalin

	Enkephalin	Product-A
0 hour	100%	0%
5 hours	81%	19%
24 hours	52%	48%

The results in Table A show that lactose reacts with enkephalin in a weakly basic solution (pH 7.9) at 40°C with the passage of time.

Test Example A:

Enkephalin (0.53 mg) was dissolved in 0.53 mL of distilled water (the enkephalin solution). β-lactose was dissolved in distilled water to make a 200 mg/mL solution (the lactose solution). The enkephalin solution (0.06 mL), the lactose solution (0.3 mL), 0.15 mL of a 200 mmol/L phosphate-citric acid buffer (pH 8.0), and 0.09 mL of distilled water were mixed in a test tube.

The obtained mixture was allowed to stand at 40°C for 24 hours. To 0.13 mL of the resulting reaction mixture was added 0.013 mL of a 200 mmol/L aqueous solution of citric acid to adjust the pH to 5.5, and the mixture was subjected to reaction at 40°C. The reaction mixture was intermittently analyzed for products obtained from the reaction by HPLC in the same manner as in the above Example A. The results are shown in Table B.

Results:

The results are shown in Table B.

Table B

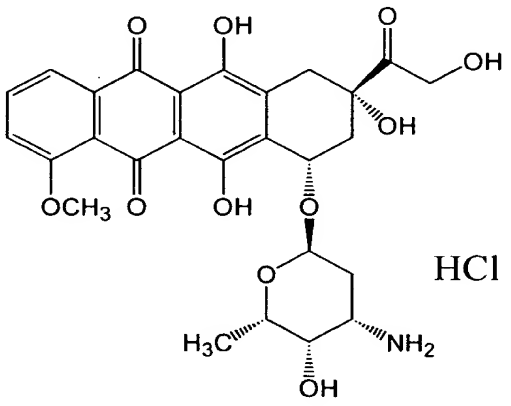
Release of Lactose Caused by pH Lowering to 5.5

	Enkephalin	Product-A
0 minute	52%	48%
10 minutes	82%	18%
30 minutes	97%	3%

As shown in Table B, the lowering of the pH of the mixture from 7.9 to 5.5 caused rapid and almost complete release of lactose from product-A.

Example B:

The structure of Doxorubicin hydrochloride (Kyowa Hakko Kogyo Co., Ltd.) is shown below.



Doxorubicin hydrochloride

Doxorubicin hydrochloride was dissolved in distilled water to make a 1 mg/mL solution (the doxorubicin solution). β-lactose (Nacalai Tesque, Inc.) was dissolved in distilled water to make a 200 mg/mL solution (the lactose solution). The doxorubicin solution (0.02 mL), the lactose solution (1 mL), and 0.98 mL of a 10 mmol/L phosphate buffer (pH 7.5) were mixed in a test tube to give a mixture having a pH of about 7.3. The test tube was put into a constant temperature water bath at 40°C

to cause reaction of Doxorubicin with lactose. The reaction mixture was sampled after 0 and 2 hours, filtered through a 0.45-μm filter, and analyzed for reaction products by high performance liquid chromatography (HPLC) under the following conditions.

Column: CAPCELL PAK C18 UG120, 4.6 x 250 mm (Shiseido Co., Ltd.)
Mobile phase:
a 10 mmol/L phosphate buffer (pH 7.0) : methanol
= 35 parts by volume : 65 parts by volume
Flow rate: 0.8 mL/minute
Detection wavelength: 254 nm

As a result, it was found that several kinds of reaction products were formed by the reaction of doxorubicin with lactose. The main reaction products were designated in order of increasing retention time on HPLC as product-D, product-C, and product-B. Doxorubicin showed the longest retention time. The results are shown in Table C.

Table C
Addition of Lactose to Doxorubicin

	Doxorubicin	Products			
		B	C	D	Others
0 hour	100%	0%	0%	0%	0%
2 hours	78%	12%	2%	4%	4%

The results in Table C show that lactose reacts with doxorubicin in a weakly basic solution (pH 7.3) at 40°C with the passage of time.

Test Example B:

Doxorubicin hydrochloride was dissolved in distilled water to make a 1 mg/mL solution (the doxorubicin solution). β-lactose (Nacalai Tesque, Inc.) was dissolved in distilled water to make a 200 mg/mL solution (the lactose solution). The doxorubicin solution (0.02 mL), the lactose solution (1 mL), and 0.98 mL of a 10 mmol/L phosphate buffer (pH 7.5) were mixed

in a test tube.

The obtained mixture was allowed to stand at 40°C for 2 hours. To 0.75 mL of the resulting reaction mixture was added 0.06 mL of a 200 mmol/L aqueous solution of citric acid to adjust the pH to 5.5, and the mixture was subjected to reaction at 40°C for 30 minutes. The reaction mixture was analyzed for products obtained from the reaction by HPLC in the same manner as in the above Example B. The results are shown in Table D.

Results:

The results are shown in Table D.

Table D

Release of Lactose Caused by pH Lowering to 5.5

	Doxorubicin	Products			
		B	C	D	Others
0 minute	78%	12%	2%	4%	4%
30 minutes	93%	0%	2%	3%	2%

As shown in Table D, the lowering of the pH of the mixture from 7.3 to 5.5 caused rapid release of lactose from product-B.

The undersigned declarant declares further that all statements made herein of his knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Executed this **7th** day of **March** , 2002.

Yasuki Kato

Yasuki KATO